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Letter to the Editor

Revisiting Claims of the Continued Absence of Functional Germline Stem Cells in Adult Ovaries

Dori C. Woods[1], Jonathan L. Tilly[*,1]

[1]Laboratory for Aging and Infertility Research, Department of Biology, Northeastern University, Boston, MA, USA

*Corresponding author: Jonathan L. Tilly, Laboratory for Aging and Infertility Research, Department of Biology, Northeastern University, Boston, MA 02115, USA. Email: j.tilly@northeastern.edu

Since the initial report in 2004 describing the existence of female germline or oogonial stem cells (OSCs) and active oogenesis in the ovaries of adult female mice,[1] more than 80 papers have been published from research groups around the world that have identified, isolated, and characterized these rare cells across diverse species, including mice, rats, pigs, cows, non-human primates, and humans[2] (see also Supporting Information Tables 1–3 in Alberico et al.[3] for a more comprehensive citation list). Being a major paradigm shift in the field of reproductive biology, the discovery of OSCs and ongoing oogenesis in adult mammalian ovaries[1] was met, not unexpectedly, with scrutiny and skepticism.[4-9] While debate over the existence of OSC has largely subsided over the years, some scientists have held fast to their longstanding opinions that this now large body of published studies on OSCs still fails to support the presence of oocyte–forming germline stem cells in adult mammalian ovaries.[10-13] Notably, one of these research groups recently concluded from single–cell RNA sequence analysis (scRNA-seq) and fluorescence–activated cell sorting (FACS) that they found no evidence of OSCs in adult human ovarian cortical tissue.[13] Given that this conclusion is discordant with more than 80 publications on OSCs to date, including at least 13 published studies from 8 different research groups which have independently isolated human OSCs for detailed characterization of germline identity, meiotic capacity, and oocyte–forming capability using a spectrum of approaches,[3,14-26] we embarked on a rigorous experimental re-assessment of this study from Wagner et al.[13] to determine the basis of this discordance.[3] These efforts uncovered several significant flaws in the analytical workflow employed by these authors, which when corrected enabled us to identify additional rare cells in the scRNA-seq datasets used by Wagner et al.[13] to dispute the existence of human OSCs. Importantly, while some of these rare cells missed by Wagner et al.[13] exhibited gene expression profiles fully consistent with those of OSCs as reported by us and others,[14-26] we also identified other rare cells similarly missed by Wagner et al.[13] with gene expression profiles consistent with non–oocyte germ cells in the early stages of meiosis-I.[3]

Shortly after the publication of our study,[3] a follow-up paper from this research group tendered a variety of explanations as to why our re-assessment of their earlier work was invalid.[27] There are five general aspects of this new publication from Lanner and Damdimopoulou that we would like to comment on. First, in making their case, Yoshihara et al.[27] begin by questioning clinical trials using mitochondria isolated from patient–matched OSCs to improve in vitro fertilization (IVF) success rates in women with a history of repeated IVF failure.[16] Inferring that these trials were driven by the discovery of human OSCs serving simply as a "*lucrative business opportunity*," Yoshihara et al.[27] selectively discuss only one of three published studies that reported the clinical outcomes of the technology, which is termed autologous germline mitochondrial energy transfer (AUGMENT). While the study discussed by these authors did not report findings supporting the use of AUGMENT in human-assisted reproduction,[28] two other clinical studies not discussed by Yoshihara et al.[27] collectively demonstrated a beneficial effect of AUGMENT on pregnancy success rates.[29,30] For the latter, 104 infertility patients, who collectively had undergone a total of 369 prior IVF cycles before enrollment in the trials, were offered the opportunity through informed consent to include AUGMENT in their next IVF attempt at clinical sites in Canada, the United Arab Emirates, and Turkey. Historical clinical pregnancy and live birth rates per cycle initiated in these patients were 5.2% (0%-11%) and 1.3% (0%-2%), respectively, with each patient having undergone at least 2, and as many as 16, prior IVF cycles. In this same patient population, a single cycle of IVF with AUGMENT yielded a clinical pregnancy rate of 26% (range of 18%-35%), with a live birth rate of 18% (range of 9%-26%) per cycle initiated. There was no maternal morbidity or mortality noted, and no still-births or neonatal deaths were reported (reviewed in[16,31]). Although these latter studies were not randomized, Fakih et al.[29] did incorporate a randomized approach termed matched best embryo selection and transfer (MBEST) in their trial. With MBEST, eggs retrieved from each patient of a subset of 25 infertility patients were allocated to IVF through either conventional intracytoplasmic sperm injection (ICSI) (n = 106 eggs) or through ICSI plus AUGMENT (n = 171 eggs). All other parameters for embryo culture and assessment were held constant until the time of embryo selection for transfer based the standard metrics of morphological grade, kinetics of early embryonic cleavage events, and preimplantation genetic screening results. The selection of embryos

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for transfer was performed blinded, such that the embryologist was unaware of whether AUGMENT was performed or not. While no differences were noted in 2-pronuclei formation rates or 5-day blastocyst development rates among the ICSI only versus ICSI plus AUGMENT groups, embryo transfer rates were 7-fold higher when AUGMENT was included compared to the rates in the ICSI only group due to vast improvements in embryo transfer selection criteria resulting from the use of AUGMENT during ICSI.[29] In evaluating the recent paper from Yoshihara et al.,[27] we note that their selective citation or discussion of certain papers that support their statements, while ignoring others that do not, is commonplace as highlighted below.

Our second point of clarification centers on why our recent re-assessment of the Wagner et al.[13] studies raised significant questions about the validity of their conclusions. Specifically, Yoshihara et al.[27] state that we believe Wagner et al.[13] "*missed OSCs due to problems with i) cell sorting, ii) numbers of cells, and iii) bioinformatic analysis*." As we document in our experimental re-assessment of this work, all of these are indeed reasons why Wagner et al.[13] failed to identify rare cells in their human ovarian cortical scRNA-datasets that are present and possess gene expression profiles consistent with OSCs as well as with early meiotic germ cells.[3] However, another major problem with the Wagner et al.[13] study, which we emphasized,[3] is that their sample preparations suffered from extensive cell damage and death. It is widely known that high cell viability is required for interpretational rigor when analyzing data derived from advanced techniques such as scRNA-seq or FACS. In addition to documenting in detail that their sample quality was compromised and how this would artifactually influence their outcomes[3] (see also Supporting Information Discussion 1 of Alberico et al.[3] for details), we provided the alert which is automatically generated by the 10xGenomics Cell Ranger software used by Wagner et al.[13] in their analysis of human unsorted ovarian cortical cells when sample quality is poor. This alert stated the following: "*the analysis detected some issues. Low Fraction Reads in Cells. Value 61.3% [GRP, 69.3%; CSP, 55.4%]. Ideal= >70%. Application performance may be affected. Many of the reads were not assigned to cell-associated barcodes. This could be caused by high levels of ambient RNA or by a significant population of cells with a low RNA content, which the algorithm did not call as cells. The latter case can be addressed by inspecting the data to determine the appropriate cell count and using force-cells*." High levels of ambient RNA and a significant population of cells with low RNA content are known signs of extensive cell damage or death, both of which would negatively impact on quality of output data obtained for downstream analysis. In addition, the compromised cellular viability associated with the Wagner et al.[13] studies should be taken into consideration by anyone interested in using a new online resource reported by Yoshihara et al.,[27] which was promoted by the authors as being a user-friendly discovery tool for exploring their human ovarian cortical cell scRNA-seq datasets.

Our third point of clarification is focused on the question of whether antibodies against the COOH-terminus of the RNA helicase, DEAD-box polypeptide 4 (DDX4), can be used to retrieve viable OSCs by FACS or magnetic-assisted cell sorting (MACS)[3,14,15,17-25] (see also Supporting Table 1 in Alberico et al.[3] for a more comprehensive citation listing). To date, more than 40 published studies have successfully used this approach to isolate OSCs from dispersed ovarian tissue of many species, including humans, and the cells obtained can be established in vitro for long-term propagation (mitotic capacity), express the expected profile of primitive germ cell–specific or –enriched genes (germline identity), can commit to and progress through meiosis (meiotic competency), and in animal models form functional oocytes that fertilize to produce viable embryos and offspring (reviewed in[2]; see also additional discussion below on approaches that have been used for testing the functionality of OSCs). Nevertheless, Yoshihara et al.[27] restate the contention of Wagner et al.[13] that DDX4 antibodies specifically bind with and isolate perivascular cells (PVCs), not OSCs. Aside from the fact that this view deviates widely from a significant body of independently corroborated studies from multiple labs which demonstrate otherwise[2] (see also Supporting Information Table 1 in Alberico et al.[3] for a more comprehensive citation listing) and was experimentally debunked,[3] Yoshihara et al.[27] failed to adequately address one of the most compelling cases raised by Alberico et al.[3] against the authors' claim that DDX4 antibodies specifically sort PVCs. In 2015, this same research group made similar claims that human OSCs do not exist and that the DDX4 antibody-based sorting strategy used to obtain OSCs is flawed.[12] In this earlier study, Lanner, Damdimopoulou, and colleagues utilized scRNA-seq to identify human ovarian cortical cells sorted as "DDX4^{+}"[12]; however, unlike what these investigators report in the Wagner et al.[13] studies, the cells obtained from human ovarian cortical biopsies identified as "DDX4^{+}" in their earlier study were a mixed pool of cell types and not simply PVCs. Further to this point, if one uses the values presented by Wagner et al.,[13] 82.5% or 34 of the 41 "DDX4^{+}" cells identified in their earlier study[12] should have been PVCs; but this was not the case. It is therefore unclear how these authors can continue to question the validity of more than 40 corroborating papers published by others that have isolated OSCs using DDX4 antibodies with FACS or MACS, based on the outcomes of a technique that they cannot achieve consistent results with across their own studies.[12,13,27] Given that half of the Wagner et al.[13] studies focused on the issue of DDX4 antibody specificity for OSC sorting, the clear discrepancy in the results of their earlier work[12] and their more recent efforts[13] with DDX4 antibody-based FACS should have warranted detailed comment and clarification in the Yoshihara et al.[27] paper, which was not provided.

Our fourth point pertains to the use of scRNA-seq as a standalone approach to identify, with confidence, specific cell types in a heterogenous tissue sample. This issue is at the heart of the argument made by Wagner et al.,[13] and then again by Yoshihara et al.,[27] that OSCs are "absent" in adult ovaries—noting that we will deal with the issue of "functional" OSCs in the second-to-last paragraph below. As emphasized in Alberico et al.,[3] technologies such as scRNA-seq are powerful tools for discovery, but these approaches have significant limitations and caveats as well. First and foremost is whether the absence of a suspected gene signature for a given cell type, in particular a rare cell type, is acceptable proof of the absence of that cell type in the sample analyzed. To this end, Wagner et al.[13] reported the identification of only 6 cell types in their tissue samples, which without question falls short of covering every cell type known to be present in the adult human ovarian cortex. However, Wagner et al.[13] targeted only OSCs as a matter of dispute, with little to no explanation as to why other cell types also absent in their analysis were missing and not similarly questioned. We also demonstrated in

our re-assessment of their datasets that expression of certain genes, which from prior studies rooted in RT-PCR or protein analysis have been consistently associated with a specific cell type, appeared far more ubiquitously across diverse cell types when scRNA-seq was employed; the "oocyte-specific" marker, *zona pellucida protein 3* (*ZP3*), was just one of many examples.[3] We further showed that genes known to be expressed by a given cell type present in their sample were not detected through scRNA-seq, even with advanced software. The most notable example of this was the absence of any cells expressing the immature oocyte-specific transcription factor, *newborn ovary homeobox* (*NOBOX*). Under the reasoning of Wagner et al.[13] with OSCs, this observation would necessitate that the authors also conclude immature oocytes are similarly absent from the ovaries of reproductive age women[3]; however, such a conclusion would be immediately dismissed by the field as erroneous and without merit. In turn, any paper submitted with such a conclusion would never be viewed as suitable for publication.

With this information as a preface, Yoshihara et al.[27] focus a significant part of their paper on whether our re-analysis of the Wagner et al.[13] datasets with more appropriate, or more current, analytical software is of value. For example, the authors state that our re-assessment of their datasets using Cell Ranger version 3.0.2 (v3), as opposed to the version 2.1.1 (v2) software employed by Wagner et al.[13] to discount the existence of OSCs in human unsorted ovarian cortical cell preparations, is problematic because Cell Ranger v3 is prone to artifact due to its enhanced detection of "*non-viable cells, cellular debris, and empty droplets*" compared with Cell Ranger v2.[27] This statement is puzzling for two reasons, the first of which is that 10xGenomics provides the appropriate tools to deal with these possibilities when using their v3 software. As just one example, Cell Ranger v3 uses an improved cell-calling algorithm based on EmptyDrops to distinguish low RNA content cells from non–viable cells and empty droplets. Indeed, our re-analysis of the Wagner et al.[13] datasets with Cell Ranger v3, along with the appropriate controls and algorithms, identified 3.4-fold more oocytes than the numbers reported by Wagner et al.[13] using v2. This illustrates the power of v3 over v2 in calling more cells of a given type or, in the case of OSCs, discovering rare cells missed when using v2.[3] Looking at this issue of claimed artifact from a different perspective, if Lanner, Damdimopoulou, and colleagues have such significant concerns with the use of Cell Ranger v3, it is unclear why these authors elected to switch from the use of v2 to v3 in the Wagner et al.[13] studies, when they moved from scRNA-seq analysis of unsorted ovarian cortical cells (use of v2) to scRNA-seq analysis of ovarian cortical cells sorted beforehand by FACS (use of v3). We also note that the same non–oocyte germline cells we identified in the Wagner et al.[13] dataset using v3 were identified a second time with an even more advanced version of Cell Ranger software (version 6.0.1),[3] which further supports that the identification of these cells missed by Wagner et al.[13] was not simply an artifact of using Cell Ranger v3. Lastly, we stand by our statement made in Alberico et al.[3] that the most rigorous approach for establishing the existence of OSCs in the ovaries of mammals is not by correlation with scRNA-seq–based gene expression profiles as the sole method. Instead, isolation of the cells for in-depth characterization through gene expression analysis coupled with numerous other analytical methods to assess meiotic potential and oocyte–forming capacity, as performed by many groups over the years[2] (see also Supporting Tables 1 and 2 in Alberico et al.[3] for a more comprehensive citation listing), is required to reach such a conclusion.

For our fifth and final point, we would like to return to an issue we touched on earlier, which hinders fair and unbiased consideration of their findings and conclusions. This pertains to a repeated lack of appropriate reference citation or discussion by Yoshihara et al.,[27] especially when such references would question, if not outright dismiss, the validity of statements made by these authors. To illustrate this point, we have selected two comments from the second-to-last paragraph of their paper to highlight here. The first concerns their statement that "*extensive work in mice has shown that generation of functional oocytes form* [sic] *regular embryonic stem cells is possible but requires closely mimicking the sequential steps of germline development in vivo*". We are pleased that the authors are convinced that functional oocytes can be formed from pluripotent stem cells using defined culture models coupled with embryo transfers[32-34]; however, we must then ask why the exact same achievement using the same technological approaches with mouse OSCs,[35] published nearly a year before submission of the Yoshihara et al.[27] paper for peer-review, was not cited or discussed by these authors? This comprehensive study from Li et al.[35] represents a key advance in once again establishing the bona fide functionality of mammalian OSCs to generate live offspring, and yet this study, and numerous other related studies (see below), were ignored by Yoshihara et al.[27] in making the broad, and scientifically unfounded, claim of a "*continued absence of functional germline stem cells in adult ovaries*".

With function defined by these authors as the ability of OSCs to differentiate into new oocytes which mature into eggs that can be fertilized to produce viable embryos and offspring, we also offer the following published studies, also not discussed by these authors, on this important topic. The first set of these studies, which utilize OSCs isolated from transgenic donor animals expressing a traceable reporter gene for intraovarian transplantation into wild-type female recipients to monitor transgenic (viz. donor OSC-derived) offspring production,[14,36-42] has served as the gold standard for functionality testing of the male equivalent cells (viz. spermatogonial stem cells or SSCs) since the 1990s.[43-45] If this approach has remained the undisputed gold standard for functional SSC identity testing for decades, it is reasonable to expect that the same approach would be equally valid, and equally accepted, for functional OSC identity testing. Moreover, if the repeated claim of Lanner and Damdimopoulou that DDX4 antibodies sort PVCs and not OSCs is true,[13,27] a logical and scientifically supported explanation is then needed from them to detail how exactly "PVCs" isolated with DDX4 antibodies are able to generate offspring after guided differentiation using 3-dimensional ovarian organoids[35] or intraovarian transplantation.[14,36-42] Results from these transplantation-based approaches, in which OSCs were isolated primarily by DDX4 antibody-based sorting, have been further bolstered by experiments with mice using inducible suicide gene technology for targeted germline cell ablation as well as genetic lineage tracing of germline cell fate in vivo.[41] These approaches, which are proven methods for evaluating the function of a given cell type to an organ or physiological process of interest in vivo, have established that new oocytes are routinely generated, de novo, in mouse ovaries during adulthood and that these new oocytes do in fact contribute to the pool of

eggs used by females under physiological conditions for offspring generation in natural mating trials.[41] Based on the collective data shown in these studies since 2009 from numerous labs employing a diverse array of technologies and analytical endpoints,[14,35-42] the concluding statement made by Yoshihara et al.[27] that "*the generation of viable offspring from DDX4 Ab+ cell derived oocytes in mice remains to be shown despite nearly two decades of research*" either highlights the authors' lack of knowledge of what has been accomplished in this field or, alternatively, represents a statement knowingly not rooted in fact. In evaluating the sequence of publications from these authors which have questioned OSCs over the years,[12,13,27] it is apparent from their literature citations that they are aware of these studies. Thus, we must conclude that this final statement by Yoshihara et al.[27] was made to, once again, propagate unfounded skepticism over the existence of OSCs for reasons that lack scientific justification.

In closing, we have always welcomed constructive discussion on the pros and cons of various methods used to study OSCs. However, these discussions must be grounded in a full and open discourse of what has been published, and not selected examples of only certain papers that are construed to sustain debate over the existence of a population of cells—viz. female germline or oogonial stem cells, which numerous labs around the world are currently working with. In short, a large volume of published work over the past 18 years has demonstrated that female germline stem cells unquestionably exist in mammals[2] (see also Supporting Information Tables 1–3 in Alberico et al.[3] for a more comprehensive citation list), and studies with rodent models have reproducibly established the functional capacity of OSCs to produce competent eggs which can be fertilized to generate embryos and offspring.[14,35-42] In addition, explorations into the potential clinical utility of OSCs,[16,23,29-31,46] which are no different from those that seek to achieve the same overarching goals of studies that focus on gametes derived from pluripotent stem cells,[32-34] should not be discouraged, repeatedly dismissed using the same arguments against the existence of OSCs,[12,13,27] or demeaned as simply a "*lucrative business opportunity*."[27] To the contrary, ongoing studies of OSCs by us and many others represent a normal, if not expected, progression of steps in hopefully meeting a key long-term objective of scientific discoveries through biomedical research—to advance new knowledge for the improvement of human health.

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Conflict of Interest

D.C.W. discloses interest in intellectual property described in U.S. Patent 8,642,329, U.S. Patent 8,647,869, U.S. Patent 9,150,830, U.S. Patent 9,525,086, and Canadian Patent 2,943,037. J.L.T. discloses interest in intellectual property described in U.S. Patent 7,195,775, U.S. Patent 7,850,984, U.S. Patent 7,955,846, U.S. Patent 8,642,329, U.S. Patent 8,647,869, U.S. Patent 8,652,840, U.S. Patent 9,150,830, U.S. Patent 9,267,111, and U.S. Patent 9,845,482, U.S. Patent 9,962,411, U.S. Patent 9,525,086, and Canadian Patent 2,943,037.

Note Added in Proof

During production of this Letter to the Editor, a new publication became available that further documents, through the use of three different antibodies, the specificity and utility of carboxy terminus-directed DDX4 antibodies to identify, sort, and isolate OSCs for detailed downstream analysis.[47] This new publication offers further evidence for the occurrence of active meiosis in adult human ovaries, as well as the impact of ovarian aging on this process.

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